

AIB1, A NOVEL STEROID RECEPTOR CO-ACTIVATOR**BACKGROUND OF THE INVENTION**

5 Breast cancer arises from estrogen-responsive breast epithelial cells. Estrogen activity is thought to promote the development of breast cancer, and many breast cancers are initially dependent on estrogen at the time of diagnosis. Anti-estrogen compositions have therefore been used to treat breast cancer.

10 A frequent mechanism of increased gene expression in human cancers is amplification, i.e., the copy number of a DNA sequence is increased, in a cancer cell compared to a non-cancerous cell. In breast cancer, commonly amplified regions are derived from 17q21, 8q24, and 11q13 which encode erbB-2, c-myc, and cyclic D1 respectively (Devilee et al., 1994, Crit. Rev. Oncog. 5:247-270). Recently, molecular cytogenetic studies have revealed the occurrence in breast cancers of additional regions of increased DNA copy number (Isola et al., Am. J. Pathol. 147:905-911, 1995; Kallioniemi et al., Proc. Natl. Acad. Sci. USA 91:2156-2160, 1994; Muleris et al., Genes Chromo. Cancer 10:160-170, 1994; Tanner et al., Cancer Research 54:4257-4260, 1994; Guan et al., Nat. Genet. 8:155-161, 1994).

20 Breast cancer is the second leading cause of cancer deaths in American women, and it is estimated that an American woman has at least a 10% cumulative lifetime risk of developing this disease. Early diagnosis is an important factor in breast cancer prognosis and affects not only survival rate, but the range of therapeutic options available to the patient. For instance, if diagnosed early, a "lumpectomy" may be performed, whereas later diagnosis tends to be associated with more invasive and traumatic surgical treatments such as radical mastectomy. The treatment of other cancers likewise is benefitted by early diagnosis, for instance the prognosis in the treatment of lung cancer, colorectal cancer and prostate cancers is greatly improved by early diagnosis. There is a need for a simple and reliable method of diagnosis of cancers in general and of breast cancer in particular. There is a need for a method of screening for compounds that inhibit the interaction between an estrogen receptor ER and an ER-dependent nuclear receptor co-activator molecule in order to identify molecules useful in research diagnosis and treatment of cancer. There is also a need for a method for identifying tamoxifen-sensitive cancer patients in order to better manage treatment. A solution to these needs would improve cancer treatment and research and would save lives.

**SUMMARY OF THE INVENTION**

35 The inventors have discovered that the AIB1 protein (Amplified In Breast Cancer-1) is a member of the Steroid Receptor Coactivator - 1 (SRC-1) family of nuclear receptor co-activators that interacts with estrogen receptors (ER) to enhance ER-dependent transcription. The inventors have further discovered that the AIB1 gene is amplified and over-expressed in certain cancers including breast cancer, and that detection of amplified AIB1 genes can therefore be used to detect

5 cancerous cells. Importantly, the inventors have also found that AIB1 amplification is not confined to breast cancer but is also found in cancers of the lung, ovary, head and neck, colon, testicles, bladder, prostate, endometrium, kidney, stomach and also in pheochromocytoma, melanoma, ductal carcinoma and carcinoid tumor. Such a finding means that AIB1 may be useful in the detection and treatment of all of the aforementioned cancers which include some of the most prevalent and deadly diseases in the western world.

10 The inventors have also discovered that AIB1 interacts with the proteins p300 and CBP, which are nuclear cofactors that interact with other nuclear factors to promote transcription (Chacravarti et al., *Nature* (383) 99-103 1996; Lundblad et al., *Nature* (374) 85-88 1995). The inventors have, furthermore, determined that in cells with stable over-expression of AIB1, there is a dramatic increase in steroid receptor activation (almost a 100-fold increase) leading to a corresponding increase in transcriptional activation. The inventors have also used monoclonal anti-AIB1 antibodies to demonstrate that AIB1 gene amplification is directly correlated with increased AIB1 expression, and that these amplified copies of the gene are expressed in physiological conditions. The inventors have found that AIB1 is the human ortholog of the mouse ER-dependent transcriptional activator p/CIP, with the proteins having an overall amino acid identity of 81.6%. These finding support the physiological role for AIB1 in cancer cells as a cofactor involved in transcriptional regulation.

20 The invention features a substantially pure DNA which includes a sequence encoding an AIB1 polypeptide, e.g., a human AIB1 polypeptide, or a fragment thereof. The DNA may have the sequence of all or part of the naturally-occurring AIB1-encoding DNA or a degenerate variant thereof. AIB1-encoding DNA may be operably linked to regulatory sequences for expression of the polypeptide. A cell containing AIB1 encoding DNA is also within the invention.

25 The invention also includes a substantially pure DNA containing a polynucleotides which hybridizes at high stringency to a AIB1-encoding DNA or the complement thereof. A substantially pure DNA containing a nucleotide sequence having at least 50% sequence identity to the full length AIB1 cDNA, e.g., a nucleotide sequence encoding a polypeptide having the biological activity of a AIB1 polypeptide, is also included.

30 The invention also features a substantially pure human AIB1 polypeptide and variants thereof, e.g., polypeptides with conservative amino acid substitutions or polypeptides with conservative or non-conservative amino acid substitutions which retain the biological activity of naturally-occurring AIB1.

35 Diagnostic methods, e.g., to identify cells which harbor an abnormal copy number of the AIB1 DNA, are also encompassed by the invention. An abnormal copy number, e.g., greater than the normal diploid copy number, of AIB1 DNA is indicative of an aberrantly proliferating cell, e.g., a steroid hormone-responsive cancer cell.

The invention also includes antibodies, e.g., a monoclonal antibody or polyclonal antisera, which bind specifically to AIB1 and can be used to detect the level of expression of AIB1 in a cell

or tissue sample. An increase in the level of expression of AIB1 in a patient-derived tissue sample compared to the level in normal control tissue indicates the presence of a cell proliferative disorder such as cancer.

Screening methods to identify compounds which inhibit an interaction of AIB1 with a steroid hormone receptor, thus disrupting a signal transduction pathway which leads to aberrant cell proliferation, is also within the invention. Proliferation of a cancer cell can therefore be reduced by administering to an individual, e.g., a patient diagnosed with a steroid-responsive cancer, a compound which inhibits expression of AIB1.

The invention also includes a knockout mutant, for example a mouse (or other mammal) from which at least one AIB1 gene has been selectively deleted from its genome. Such a mouse is useful in research, for instance, the phenotype gives insight into the physiological role of the deleted gene. For instance the mutant may be defective in specific biochemical pathways; such a knockout mutant may be used in complementation experiments to determine the role of other genes and proteins to determine if any such genes or proteins complement for the deleted gene.

Homozygous and heterozygous mutants are included in this aspect of the invention.

The present invention also includes a mutant organism, for example a mammal such as a mouse which contains more than the normal number of AIB1 genes in its genome. Such a mouse may contain additional copies of the AIB1 gene integrated into its chromosomes, for instance in the form of a pro-virus, or may carry additional copies on extra-chromosomal elements such as plasmids. Such a mutant mouse is useful for research purposes, to elucidate the physiological or pathological role of AIB1. For instance, the role of AIB1 expression as cause or effect in cancers may be investigated by including or transplanting tumors into such mutants, and comparing such mutants with normal mice having the same cancer.

The present invention also includes a mutant organism, for example a mammal, e.g. a mouse, that contains, either integrated into a chromosome or on a plasmid, at least one copy of the AIB1 gene driven by a non-native promoter. Such a promoter may be constitutive or may be inducible. For instance, the AIB1 gene may be operatively linked to a mouse mammary tumor virus (MMTV) promoter or other promoter from a mammalian virus allowing manipulation of AIB1 expression. Such a mutant would be useful for research purposes to determine the physiological or pathological role of AIB1. For instance, over or under expression could be affected and physiological effects observed.

The invention also includes methods for treatment of cancers that involve functions of or alterations in the signaling pathways that use p300 and/or CBP as signal transducing molecules. The treatments of the invention involve targeting of the AIB1 protein or AIB1 gene to enhance or reduce interaction with p300 and/or CBP proteins. For instance, the AIB1 gene sequence as disclosed herein may be used to construct an anti-sense nucleotide. An anti-sense RNA may be constructed that is anti-parallel and complementary to the AIB1 transcript (or part thereof) and which will therefore form an RNA-RNA duplex with the AIB1 transcript, preventing transcription

and expression of AIB1. Alternatively, treatments may comprise contacting an AIB1 protein with a molecule that specifically binds to the AIB1 molecule *in vivo*, thereby interfering with AIB1 binding with other factors such as p300 or CBP. Such processes are designed to inhibit signal transduction pathways involving AIB1, p300, CBP and other factors and therefore inhibit cancer cell proliferation that is effected via these pathways. As explained in more detail below, AIB1 overexpression results in increased ER-dependent transcriptional activity which confers a growth advantage upon AIB1 amplification-bearing clones during the development and progression of estrogen-dependent cancers.

Compounds which inhibit or disrupt the interaction of an AIB1 gene product with a steroid hormone receptor, e.g., ER, are useful as anti-neoplastic agents for the treatment of patients suffering from steroid hormone-responsive cancers such as breast cancer, ovarian cancer, prostate cancer, and colon cancer.

AIB1 polypeptides or peptide mimetics of such polypeptides, e.g., those containing domains which interact with steroid hormone receptors, can be administered to patients to block the interaction of endogenous intracellular AIB1 and a steroid hormone receptor, e.g., ER in an aberrantly proliferating cell. It is likely that AIB1 interacts with a wide range of human transcriptional factors and that regulation of such interactions will have important therapeutic applications.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### SEQUENCE LISTING

The nucleic acid and amino acid sequences listed in the accompanying Sequence Listing are shown using standard letter abbreviations for nucleotide bases and three-letter code for amino acids. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood to be included by any reference to the displayed strand.

SEQ. I.D. No. 1 shows the nucleic acid sequence of the human AIB1 cDNA and the corresponding amino acid sequence.

SEQ. I.D. No. 2 shows the amino acid sequence of the Per/Arnt/Sim (PAS) domain of AIB1.

SEQ. I.D. No. 3 shows the amino acid sequence of the basic helix-loop-helix domain (bHLH) of AIB1.

SEQ. I.D. No. 4 shows the amino acid sequence of the human AIB1 protein.

SEQ. I.D. No. 5 shows the nucleic acid sequence of primer N8F1.

SEQ. I.D. No. 6 shows the nucleic acid sequence of the forward primer designed from the 5' sequence of pCMVSPORT-B11, PM-U2.

SEQ. I.D. No. 7 shows the nucleic acid sequence of the reverse primer designed from the 5' sequence of pCMVSPORT-B11, PM-U2.

SEQ. I.D. No. 8 shows the amino acid sequence of the ER-interacting domain of AIB1.

SEQ. I.D. No. 9 shows the nucleic acid sequence of pCIP, the mouse ortholog of AIB1 and the amino acid sequence for this gene.

SEQ. I.D. No. 10 shows the nucleic acid sequence of the forward primer AIB1/mESTF1 used to screen mouse BAC.

SEQ. I.D. No. 11 shows the nucleic acid sequence of the reverse primer AIB1/mESTR1 used to screen mouse BAC.

SEQ. I.D. No. 12 shows the amino acid sequence of pCIP, the mouse ortholog of AIB1.

### Brief Description of the Drawings

#### FIGURES

Fig. 1A is a diagram of an amino acid sequence of full length AIB1 in which residues highlighted in black are identical in AIB1, TIF2 and SRC1. Residues identical with TIF2 (GenBank accession number X97674) or SRC-1 (GenBank accession number U59302) are highlighted in grey or boxed, respectively.

Fig. 1B is a diagram showing the structural features of AIB1. The following domains are indicated: bHLH domain, PAS domains (with the highly conserved PAS A and B regions shown in dark gray), S/T (serine/threonine)-rich regions, and a group of charged residues (+/-). A glutamine-rich region and polyglutamine tract are also indicated. The numbers beneath the diagram indicate the location (approximate residue number) of the domain with respect to the amino acid sequence shown in Fig. 1A. The alignment was generated using DNASTAR software.

Fig. 2 is a photograph of a Northern blot analysis showing increased expression of AIB1 in the cell lines BT-474, ZR-75-1, MCF7, and BG-1.

Fig. 3 is a bar graph showing that the addition of full length AIB1 DNA to a cell resulted in an increase of estrogen-dependent transcription from an ER reporter plasmid. COS-1 cells were transiently transfected with 250 ng ER expression vector (pHEGO-hyg), 10 ng of luciferase reporter plasmid (pGL3.luc.3ERE or 10 ng pGL3 lacking ERE) and increasing amounts of pcDNA3.1-AIB1 and incubated in the absence (open bars) or presence of 10 nM 17 $\beta$ -estradiol (E2, solid bars) or 100 nM 4-hydroxytamoxifen (hatched bars). Luciferase activity was expressed in relative luminescence units (RLU). The data are the mean of three determinations from one of four replicate experiments. Error bars indicate one standard deviation.

Fig. 4 is a schematic diagram comparing the DNA and protein structures of pCIP (the mouse ortholog of AIB1) and the human AIB1; exons are shown as black boxes.

Fig. 5 is a table showing the introns and exons of the mouse AIB1 gene (pCIP). The "Exon" column refers to the number of the exon; "cDNA bp 5'-exon" refers to the nucleotide position in the mouse cDNA sequence for the 5' exon. "3' intron splice cite" refers to the last few nucleotides of the 3' position of the intron. "Exon sequence" refers to the exon itself. "5' intron" refers to the adjacent intron reading from the exon into the splice donor elinucleotides (usually GT).

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Fig. 6 is a table showing the introns and exons of the human AIB1 gene. The "Exon" column refers to the number of the exon; "cDNA bp 5'-exon" refers to the nucleotide position in the mouse cDNA sequence for the 5' exon. "3' intron splice cite" refers to the last few nucleotides of the 3' position of the intron. "Exon sequence" refers to the exon itself. "5' intron" refers to the adjacent intron reading from the exon into the splice donor nucleotides (usually GT).

#### DETAILED DESCRIPTION

The invention is based on the discovery of a novel gene, amplified in breast cancer-1 (AIB1), which is overexpressed in breast cancer. AIB1 has the structural features of a co-activator of the steroid hormone receptor family. The steroid hormone estrogen and other related steroid hormones act on cells through specific steroid receptors.

Members of the steroid receptor coactivator (SRC) family of transcriptional co-activators interact with nuclear hormone receptors to enhance ligand-dependent transcription. AIB1 is a novel member of the SRC family which was found to be overexpressed in breast cancers. The AIB1 gene is located at human chromosome 20q. High-level AIB1 amplification and overexpression were observed in several estrogen receptor (ER) positive breast and ovarian cancer cell lines, as well as in uncultured breast cancer specimens. AIB1 amplification is not confined to breast cancer but is also found in cancers of the lung, ovary, head and neck, colon, testicles, bladder, prostate, endometrium, kidney, stomach and also in pheochromocytoma, melanoma, ductal carcinoma and carcinoid tumor.

Transfection of AIB1 into cells resulted in marked enhancement of estrogen-dependent transcription. These observations indicated that AIB1 functions as a co-activator of steroid hormone receptors such as ER (including estrogen receptor  $\alpha$  (ER $\alpha$ ) and estrogen receptor  $\beta$  (ER $\beta$ )), androgen receptor (e.g., expressed in prostate cells), retinoid receptor (e.g., isoforms  $\alpha$ ,  $\gamma$ , and retinoid X receptor (RXR)), progesterone receptor (e.g., expressed in breast cells), mineralocorticoid receptor (implicated in salt metabolism disorders), vitamin D receptor (implicated in calcium metabolism disorders), thyroid hormone receptor (e.g., thyroid hormone receptor  $\alpha$ ), or glucocorticoid receptor (e.g., expressed in spleen and thymus cells). The altered expression of AIB1 contributes to the initiation and progression of steroid hormone-responsive cancers by increasing the transcriptional activity of the steroid receptor.

A substantially pure DNA which includes an AIB1-encoding polynucleotides (or the complement thereof) is claimed. By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the AIB1 gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote at a site other than its natural site; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a

recombinant DNA which is part of a hybrid gene encoding an additional polypeptide sequence.

Preferably, the polypeptide includes a Per/Arnt/Sim (PAS) domain

(LLQALDGLFVFNDRDGNIVFVSENVNTQYLQYKQEDLVNTSVYNILHEEDRKDFLKNLPKST  
VNGVSWTNETQRQKSHTFNCRMLMKTPHDILEDINASPEMRQRYETMQCFALSQPRAMME  
5 EGEDLQSCMICVARRITTGERTFPSNPESFITRHDLSGKVVNIDTNSLRSSMRPGFEDIIRRCIQ  
; SEQ. I.D. NO. 2) and/or a basic helix-loop-helix

(bHLH) domain (RKRKLPCDTPGQGLTCSGEKRRREQESKYIEELAEELISANLSDIDNFNVKPD  
KCAILKETVRQIRQIKEQGKT; SEQ. I.D. NO. 3); more preferably, the AIB1 polypeptide  
includes the amino acid sequence of the entire naturally-occurring AIB1 protein (Fig. 1; SEQ. I.D.  
10 NO. 4). Preferably, the peptide includes an ER-interacting domain of AIB1 (e.g., a domain  
comprising approximately amino acids 300 to 1250:

CIQRFFSLNDGQSWSQKRHYQEAYLNGHAETPVYRFSADGTIVTAQTKSKLF  
RNPVTNDRHGFVSTHFLQREQNGYRPNPNPVGQGIRPPMAGCNSSVGGMSMS  
PNQGLQMPSSRAYGLADPSTTGQMSGARYGGSSNIASLTGPGMQSPSSYQNNNYGLNMSS  
15 PPHGSPGLAPNQNNIMISPRNRGSPKIAHQFSPVAGVHSPMASSGNTGNHSFSSSSLSALQAI  
SEGVGTSLSTLSSPGPKLDNSPNMNITQPSKVSNDQSKSPLGFYCDQNPVESSMCQSNRSDH  
LSDKESKESSVEGAENQRGPLESKGHKLLQLLTCSDDRGHSSLTNSPLDSSCKESSVSVTS  
PSGVSSSTSGGVSSSTSNMHGSLLEKHLRILHKLQNGNSPAEVAKITAEATGKDTSSITSCGD  
GNVVKQEQLSPKKKENNALLRYLLDRDDPSDALSKELQPQVEGVDNKMSQCTSSTIPSSSQE  
20 KDPKIKTETSEEGSGDLNDLDAILDLTSSDFYNNSISSNGSHLGTKQQVFQGTNSLGLKSSQ  
SVQSIRPPYNRAVSLDSPVSVGSSPPVKNISAFPMPLPKQPMLGGNPRMMDSQENYGSSMGGP  
NRNVTVTQTPSSGDWGLPNSKAGRMEPMNSNSMGRPGGDYNTSLPRPALGGS IPTLPLRSN  
SIPGARPVLLQQQQMLQMRPGEIPMGMGANPYGQAAASNQLGSWPDGMLSMEQVSHGTQ  
NRPLLRNSLDDLVGPPSNLEGQSDERALLDQLHTLLSNTDATGLEEIDRALGIPELVNQGQA  
25 LEPKQDAFQGQEAAMMDQKAGLYGQTYPAQGPPMQGGFHLQGSFNSMMNQMNQQ  
GNFPLQGMHPRANIMRPTNTPKQLRMQLQQRLLQGSFNSMMNQMNQQ  
VMRPMMPQGGFLNAQMVAQRSRELLSHHFRQQRVAMMMQQQQQQQ (SEQ. I.D. NO.

8). A cell containing substantially purified AIB1-encoding DNA is also within the invention.

The invention also includes a substantially pure DNA which contains a polynucleotide which  
30 hybridizes at high stringency to an AIB1 cDNA having the sequence of SEQ. I.D. NO. 1, or the  
complement thereof and a substantially pure DNA which contains a nucleotide sequence having at  
least 50% (for example at least 75%, 90%, 95%, or 98-100%) sequence identity to SEQ. I.D. NO.  
1, provided the nucleotide sequence encodes a polypeptide having the biological activity of a AIB1  
polypeptide. By "biological activity" is meant steroid receptor co-activator activity. For example,  
35 allelic variations of the naturally-occurring AIB1-encoding sequence (SEQ. I.D. NO. 1) are  
encompassed by the invention. Sequence identity can be determined by comparing the nucleotide  
sequences of two nucleic acids using the BLAST sequence analysis software, for instance, the

NCBI gapped BLAST 2.0 program set to default parameters. This software is available from The National Center for Biotechnology Information ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)).

Hybridization is carried out using standard techniques such as those described in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, (1989). "High stringency" refers to DNA hybridization and wash conditions characterized by high temperature and low salt concentration, e.g., wash conditions of 65° C at a salt concentration of approximately 0.1 X SSC. "Low" to "moderate" stringency refers to DNA hybridization and wash conditions characterized by low temperature and high salt concentration, e.g. wash conditions of less than 60° C at a salt concentration of at least 1.0 X SSC. For example, high stringency conditions may include hybridization at about 42°C, and about 50% formamide; a first wash at about 65°C, about 2X SSC, and 1% SDS; followed by a second wash at about 65°C and about 0.1% x SSC. Lower stringency conditions suitable for detecting DNA sequences having about 50% sequence identity to an AIB1 gene are detected by, for example, hybridization at about 42°C in the absence of formamide; a first wash at about 42°C, about 6X SSC, and about 1% SDS; and a second wash at about 50°C, about 6X SSC, and about 1% SDS.

A substantially pure DNA including (a) the sequence of SEQ ID NO. 1 or (b) a degenerate variant thereof is also within the invention. The AIB1-encoding DNA is preferably operably linked to regulatory sequences (including, e.g., a promoter) for expression of the polypeptide.

By "operably linked" is meant that a coding sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

The invention also includes a substantially pure human AIB1 polypeptide or fragment thereof. The AIB1 fragment may include an ER-interaction domain such as one having the amino acid sequence of SEQ. I.D. NO. 8. Alternatively, the fragment may contain the amino acid sequence of SEQ. I.D. NOS. 2, 3, or 4.

Screening methods to identify candidate compounds which inhibit estrogen-dependent transcription, AIB1 expression, or an AIB1/ER interaction (and as a result, proliferation of steroid hormone-responsive cancer cells) are within the scope of the invention. For example, a method of identifying a candidate compound which inhibits ER-dependent transcription is carried out by contacting the compound with an AIB1 polypeptide and determining whether the compound binds to the polypeptide. Binding of the compound to the polypeptide indicates that the compound inhibits ER-dependent transcription, and in turn, proliferation of steroid hormone-responsive cancer cells. Preferably, the AIB1 polypeptide contains a PAS domain or a bHLH domain. Alternatively, the method is carried out by contacting the compound with an AIB1 polypeptide and an ER polypeptide and determining the ability of the compound to interfere with the binding of the ER polypeptide with the AIB1 polypeptide. A compound which interferes with an AIB1/ER interaction inhibits ER-dependent transcription.



A method of screening a candidate compound which inhibits an interaction of an AIB1 polypeptide with an ER polypeptide in a cell includes the steps of (a) providing a GAL4 binding site linked to a reporter gene; (b) providing a GAL4 binding domain linked to either (i) an AIB1 polypeptide or (ii) an ER polypeptide; (c) providing a GAL4 transactivation domain II linked to the ER polypeptide if the GAL4 binding domain is linked to the AIB1 polypeptide or linked to the AIB1 polypeptide if the GAL4 binding domain is linked to the ER polypeptide; (d) contacting the cell with the compound; and (e) monitoring expression of the reporter gene. A decrease in expression in the presence of the compound compared to that in the absence of the compound indicates that the compound inhibits an interaction of an AIB1 polypeptide with the ER polypeptide.

Diagnostic methods to identify an aberrantly proliferating cell, e.g., a steroid hormone-responsive cancer cell such as a breast cancer cell, ovarian cancer cell, or prostate cancer cell, are also included in the invention. For example, a method of detecting an aberrantly proliferating cell in a tissue sample is carried out by determining the level of AIB1 gene expression in the sample. An increase in the level of gene expression compared to that in a normal control tissue indicates the presence of an aberrantly proliferating cell. AIB1 gene expression is measured using an AIB1 gene-specific polynucleotides probe, e.g. in a Northern assay or polymerase chain reaction (PCR)-based assay, to detect AIB1 mRNA transcripts. AIB1 gene expression can also be measured using an antibody specific for an AIB1 gene product, e.g., by immunohistochemistry or Western blotting.

Aberrantly proliferating cells, e.g., cancer cells, in a tissue sample may be detected by determining the number of cellular copies of an AIB1 gene in the tissue. An increase in the number of gene copies in a cell of a patient-derived tissue, compared to that in normal control tissue indicates the presence of a cancer. A copy number greater than 2 (the normal diploid copy number) is indicative of an aberrantly proliferative cell. Preferably, the copy number is greater than 5 copies per diploid genome, more preferably 10 copies, more preferably greater than 20, and most preferably greater than 25 copies. An increase in copy number compared to the normal diploid copy number indicates that the tissue sample contains aberrantly proliferating steroid hormone-responsive cancer cells. AIB1 copy number is measured by fluorescent *in situ* hybridization (FISH), Southern hybridization techniques, and other methods well known in the art (Kallioniemi et al., *PNAS* 91: 2156-2160 (1994); Guan et al., *Nature Genetics* 8: 155-161 (1994); Tanner et al., *Clin. Cancer Res.* 1: 1455-1461 (1995); Guan et al., *Cancer Res.* 56: 3446-3450 (August 1996); Anzick et al., *Science* 277: 965-968 (August 1997)).

Aberrantly proliferating cells can also be identified by genetic polymorphisms in the polyglutamine tract of AIB1, e.g., variations in the size of this domain which alter AIB1 co-activator activity.

The invention also includes methods of treating a mammal, e.g., a human patient. For example, a method of reducing proliferation of a steroid hormone-responsive cancer cell, e.g., an estrogen-responsive breast cancer cell, in a mammal is carried out by administering to the mammal a compound which inhibits expression of AIB1. The compound reduces transcription of AIB1-

encoding DNA in the cell. Alternatively, the compound reduces translation of an AIB1 mRNA into an AIB1 gene product in the cell. For example, translation of AIB1 mRNA into an AIB1 gene product is inhibited by contacting the mRNA with antisense polynucleotides complementary to the AIB1 mRNA.

5 A method of inhibiting ER-dependent transcription in a breast cell of a mammal is carried out by administering an effective amount of an AIB1 polypeptide or a peptide mimetic thereof to the mammal. Preferably, the polypeptide inhibits an AIB1/ER interaction; more preferably, the polypeptide contains an ER-interacting domain; a PAS domain or a bHLH domain of AIB1. By binding to ER, such a polypeptide inhibits binding of AIB1 to ER, thereby inhibiting ER-dependent transcription.

10 The invention also includes antibodies, e.g., a monoclonal antibody or polyclonal antisera, which bind specifically to AIB1. The term "antibody" as used in this invention includes whole antibodies as well as fragments thereof, such as Fab, Fab', F(ab')<sub>2</sub>, and Fv which bind to an AIB1 epitope. These antibody fragments are defined as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')<sub>2</sub>, the fragment of the antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')<sub>2</sub>, a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain, the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule. Methods of making these fragments are routine.

25 Also within the invention is a method of identifying a tamoxifen-sensitive patient (one who is likely to respond to tamoxifen treatment by a reduction in rate of tumor growth) wherein the method includes the steps of (a) contacting a patient-derived tissue sample with tamoxifen; and (b) determining the level of AIB1 gene expression or amplification in the sample. An increase in the level of expression or gene copy number compared to the level or cellular copy number in normal control tissue indicates that the patient is tamoxifen-sensitive.

30 AIB1 gene expression is measured using an AIB1 gene-specific polynucleotide probe, e.g., in a Northern blot or PCR-based assay to detect AIB1 mRNA transcripts or in a Southern blot or FISH assay to detect amplification of the gene (which correlates directly with AIB1 gene expression). Alternatively, AIB1 gene expression is measured by detecting an AIB1 gene product, e.g., using an AIB1-specific antibody.

Transgenic mammals, e.g., mice, which overexpress an AIB1 gene product, e.g., by virtue of harboring multiple copies of AIB1-encoding DNA, are also within the invention.

"Transgenic" as used herein means a mammal which bears a transgene, a DNA sequence which is inserted by artifice into an embryo, and which then becomes part of the genome of the mammal that develops from that embryo. Any non-human mammal which may be produced by transgenic technology is included in the invention; preferred mammals include, mice, rats, cows, pigs, sheep, goats, rabbits, guinea pigs, hamsters, and horses.

By "transgene" is meant DNA which is partly or entirely heterologous (i.e., foreign) to the transgenic mammal, or DNA homologous to an endogenous gene of the transgenic mammal, but which is inserted into the mammal's genome at a location which differs from that of the natural gene.

Also within the invention is a knockout mutant, for instance a knockout mouse wherein the mouse has had at least one copy of the AIB1 gene (also called the pCIP gene in mice) deleted from its genome. Such a knockout mutant would be useful in research, for instance the phenotype gives insight into the physiological role of AIB1. Complementation experiments using such a knockout mutant can be used to identify other genes and proteins that make up for the lack of AIB1 in the mutant to restore wild-type phenotype.

Also within the invention is a mutant, such as a mouse, which contains more than the normal number of copies of the AIB1 (pCIP) gene, either integrated into a chromosome, for instance as a pro-virus, or in an extra-chromosomal element, such as on a plasmid.

Also within the invention is a mutant, for example, a mouse, which contains the AIB1 (pCIP) gene driven by a non-native promoter, such as a constitutive or an inducible promoter, such as the mouse mammary tumor virus (MMTV) promoter.

The invention also includes methods of treatment for cancers the growth of which involves alternations of signaling pathways involving p300 and/or CBP. For example, AIB1 (pCIP) may be contacted with a molecule that binds to AIB1 and inhibits AIB1's interaction with p300, thereby disrupting signaling of this pathway and reducing transcription of molecules whose transcription is positively regulated by this pathway; thereby reducing tumor growth.

#### Example 1: Cloning and Expression of AIB1

##### A. Cloning of AIB1

Chromosome microdissection and hybrid selection techniques were used to isolate probes and clone gene sequences which map to chromosome 20q, one of the recurrent sites of DNA amplification in breast cancer cells identified by molecular cytogenetics (Kallioniemi et al., *PNAS* 91: 2156-2160 (1994); Guan et al., *Nature Genetics* 8: 155-161 (1994); Tanner et al., *Clin. Cancer Res.* 1: 1455-1461 (1995); Guan et al., *Cancer Res.* 56: 3446-3450 (August 1996); Anzick et al., *Science* 277: 965-968 (August 1997)). AIB1 is a member of the SRC-1 family of nuclear receptor (NR) co-activators. AIB1 functions to enhance ER-dependent transcription. SRC-1 and the closely

related TIF2 are steroid receptor co-activators with an affinity for NRs. The mouse ortholog of human AIB1 is called pCIP. In this application pCIP and AIB1 will be used synonymously unless the contrary is clearly expressed.

To characterize AIB1, the full length cDNA was cloned and sequenced. An AIB1 specific primer N8F1 (5'-TCATCACTTCCGACAACAGAGG-3'; SEQ. I.D. NO. 5) was biotinylated and used to capture cDNA clones from a human lung cDNA library (Gibco, BRL) using the GENETRAPPER cDNA Positive Selection System (Gibco, BRL). The largest clone (5.8 kb), designated pCMVSPORT-B11, was selected for sequence analysis. To obtain full-length AIB1-encoding DNA, a random-primed library from BT-474 was constructed in bacteriophage  $\lambda$ -Zap (Stratagene) and hybridized with a 372 bp  $^{32}$ P-labeled PCR product amplified from a human spleen cDNA library using primers designed from the 5' sequence of pCMVSPORT-B11, PM-U2 (5'-CCAGAAACGTCACTATCAAG-3', forward primer; SEQ. I.D. NO. 6) and B11-11RA (5'-TTACTGGAACCCCCATACC-3', reverse primer; SEQ. I.D. NO. 7). Plasmid rescue of 19 positive clones yielded a clone, pBluescript-R22, which overlapped pCMVSPORT-B11 and contained the 5' end of the coding region. To generate a full length AIB1 clone, the 4.85 kb HindIII/XhoI fragment of pCMVSPORT-B11 was subcloned into HindIII/XhoI sites of pBluescript-R22. The 4.84 kb NotI/NheI fragment of the full length clone containing the entire coding region was then subcloned into the NotI/XbaI sites of the expression vector, pcDNA3.1 (Invitrogen), generating pcDNA3.1-AIB1.

The cloned DNA sequence (SEQ. I.D. No. 1) revealed an open reading frame (beginning at the underlined "ATG") encoding a protein of 1420 amino acids with a predicted molecular weight of 155 kDa (Fig. 1A). Database searches with BLASTP identified a similarity of AIB1 with TIF2 (45% protein identity) and SRC-1 (33% protein identity). Like TIF2 and SRC-1, AIB1 contains a bHLH domain preceding a PAS domain, serine/threonine-rich regions, and a charged cluster (Fig. 1B). There is also a glutamine-rich region which, unlike SRC-1 and TIF2, contains a polyglutamine tract (Fig. 1B). The polyglutamine tract of AIB1 is subject to genetic polymorphism. Variations in the size of this domain alter AIB1 co-activator activity.

#### B. Expression of AIB1

Amplification and expression of AIB1 in several ER positive and negative breast and ovarian cancer cell lines was examined. Established breast cancer cell lines used in the experiments described below (see, e.g., Fig. 2) were obtained from the American Type Culture Collection (ATCC): BT-474, MCF-7, T-47D, MDA-MB-361, MDA-MB-468, BT-20, MDA-MB-436, and MDA-MB-453; the Arizona Cancer Center (ACC): UACC-812; or the National Cancer Institute (NCI): ZR75-1.

AIB1 gene copy number was determined by FISH. For FISH analysis, interphase nuclei were fixed in methanol:acetic acid (3:1) and dropped onto microscope slides. AIB1 amplification was detected in the breast cancer cell line ZR75-1, the ovarian cancer cell line BG-1, and two

uncultured breast cancer samples. Intra-chromosomal amplification of AIB1 was apparent in metaphase chromosomes of ZR75-1 and BG1. Numerous copies of AIB1 were resolved in the adjacent interphase nuclei. Extrachromosomal copies (e.g., in episomes or double minute chromosomes) of AIB1 have also been detected. The Spectrum-Orange (Vysis) labeled AIB1 P1 probe was hybridized with a biotinylated reference probe for 20q11 (RMC20P037) or a fluorescein labeled probe for 20p (RMC20C039).

High level amplification of AIB1 (greater than 20 fold), similar to that observed in BT-474 and MCF-7, was seen in two additional ER-positive cell lines, breast carcinoma ZR75-1, and ovarian carcinoma BG-1 (see Fig. 2). Interphase FISH studies demonstrated that amplification of chromosome 20q in breast cancer is complex, involving several distinct variably co-amplified chromosomal segments derived from 20q11, 20q12, and 20q13. Probes for the 20q11 and 20q13 regions of amplification did not detect amplification in ZR75-1 and BG-1, suggesting that amplification of AIB1 (which maps to 20q12) occurred independently in these cell lines.

To determine if AIB1 amplification also occurred in uncultured cells from patient biopsies, breast cancer specimens were screened for AIB1 amplification by interphase FISH. In two of 16 specimens analyzed, high AIB1 copy number (up to 25 copies/cell) was detected. Both tumor specimens tested came from post-menopausal patients and were ER/PR positive. One of the specimens was obtained from a metastatic tumor of a patient who subsequently responded favorably to tamoxifen treatment.

AIB1 expression was also examined in cells with and without AIB1 amplification and compared to expression of ER, SRC-1 and TIF2 by Northern blotting. In accordance with its amplification status, AIB1 was highly overexpressed in BT-474, MCF-7, ZR75-1, and BG-1 (Fig. 2). Three of the four cell lines exhibiting AIB1 overexpression also demonstrated prominent ER expression, while two others displayed lower but detectable ER expression (BT-474 and BT-20). Fig. 2 also shows that the expression of TIF2 and SRC-1 remained relatively constant in all cell lines tested. Taken together, these observations demonstrate that AIB1 amplification is associated with significant overexpression of AIB1 gene product. The correlation of elevated AIB1 expression with ER positivity in tumors indicates that AIB1 is a component of the estrogen signaling pathway, the amplification of which is selected during cancer development and progression.

To determine whether expression of AIB1 increases ER ligand-dependent transactivation, transient transfection assays were performed. The effect of increasing levels of AIB1 on transcription of an ER dependent reporter was measured. The results demonstrated that co-transfection of AIB1 led to a dose dependent increase in estrogen-dependent transcription (Fig. 3). This effect was not observed when the estrogen antagonist, 4-hydroxytamoxifen (4-OHT), was substituted for  $17\beta$ -estradiol or when the estrogen response element (ERE) was removed from the reporter plasmid (Fig. 3). A modest increase in basal transcription levels was observed with higher concentrations of AIB1 even in the absence of an ERE suggesting that AIB1 may have an intrinsic

transactivation function. These results demonstrate that, like the closely related TIF2 and SRC-1, AIB1 functions as an ER co-activator.

#### Example 2: Characterization of AIB1

##### 5 A. Functional Domains of AIB1

TIF-2, SRC-1, and AIB1 are characterized by highly conserved N-terminal bHLH and PAS domains. The PAS region functions as a protein dimerization interface in the mammalian aryl hydrocarbon receptor and the aryl hydrocarbon receptor nuclear transporter proteins, as well as the *Drosophila* transcription factors *sim* and *per*. The PAS region (SEQ. I.D. NO. 2) of AIB1  
10 functions as a protein interaction domain, mediating binding between AIB1 and other proteins. However, steroid hormone activators lacking the PAS domain are capable of interacting with nuclear steroid hormone receptors. The highly conserved bHLH domain (SEQ. I.D. NO. 3) participates in protein interactions which mediate or modulate transmission of the hormone signal to the transcriptional apparatus. The ER-interacting domain (SEQ. I.D. NO. 8) mediates binding of  
15 AIB1 with a steroid hormone receptor protein.

AIB1 also interacts with the transcriptional integrators CREB binding protein (CBP) and p300. These transcriptional integrators interact directly with the basal transcriptional machinery. The CBP/p300 receptor association domain of AIB1 does not encompass the bHLH/PAS regions.

##### B. Purification of Gene Products

20 DNA containing a sequence that encodes part or all of the amino acid sequence of AIB1 can be subcloned into an expression vector, using a variety of methods known in the art. The recombinant protein can then be purified using standard methods. For example, a recombinant polypeptide can be expressed as a fusion protein in procaryotic cells such as *E. coli*. Using the maltose binding protein fusion and purification system (New England Biolabs), the cloned human  
25 cDNA sequence is inserted downstream and in frame of the gene encoding maltose binding protein (malE). The malE fusion protein is overexpressed in *E. coli* and can be readily purified in quantity. In the absence of convenient restriction sites in the human cDNA sequence, PCR can be used to introduce restriction sites compatible with the pMalE vector at the 5' and 3' end of the cDNA fragment to facilitate insertion of the cDNA fragment into the vector. Following expression  
30 of the fusion protein, it can be purified by affinity chromatography. For example, the fusion protein can be purified by virtue of the ability of the maltose binding protein portion of the fusion protein to bind to amylase immobilized on a column.

To facilitate protein purification, the pMalE plasmid contains a factor Xa cleavage site upstream of the site into which the cDNA is inserted into the vector. Thus, the fusion protein  
35 purified as described above can be cleaved with factor Xa to separate the maltose binding protein portion of the fusion protein from recombinant human cDNA gene product. The cleavage products can be subjected to further chromatography to purify recombinant polypeptide from the maltose binding protein. Alternatively, an antibody specific for the desired recombinant gene product can

be used to purify the fusion protein and/or the gene product cleaved from the fusion protein. Many comparable commercially available fusion protein expression systems can be utilized similarly.

AIB1 polypeptides can also be expressed in eucaryotic cells, e.g., yeast cells, either alone or as a fusion protein. For example, a fusion protein containing the GAL4 DNA-binding domain or activation domain fused to a functional domain of AIB1, e.g., the PAS domain, the bHLH domain, or the ER-interacting domain, can be expressed in yeast cells using standard methods such as the yeast two hybrid system described below. Alternatively, AIB1 polypeptides can be expressed in COS-1 cells using methods well known in the art, e.g., by transfecting a DNA encoding an AIB1 polypeptide into COS-1 cells using, e.g., the Lipofectamine transfection protocol described below, and culturing the cells under conditions suitable for protein expression.

### Example 3: Detection of AIB1

#### **A. Detection of Nucleotides Encoding AIB1**

Determination of gene copy number in cells of a patient-derived sample is known in the art. For example, AIB1 amplification in cancer-derived cell lines as well as uncultured breast cancer cells was carried out using bicolor FISH analysis as follows. A genomic P1 clone containing AIB1 was labeled with Spectrum Orange-dUTP (Vysis) using the BioPrime DNA Labeling System (Gibco BRL). A 20q11 P1 clone was labeled with Biotin-16-dUTP (BMB) using nick translation. Fluorescent images were captured using a Zeiss axiophot microscope equipped with a CCD camera and IP Lab Spectrum software (Signal Analytics). Interphase FISH analysis of uncultured breast cancer samples was performed using known methods (Kallioniemi et al., *PNAS* 91: 2156-2160 (1994); Guan et al., *Nature Genetics* 8: 155-161 (1994); Tanner et al., *Clin. Cancer Res.* 1: 1455-1461 (1995); Guan et al., *Cancer Res.* 56: 3446-3450 (August 1996); Anzick et al., *Science* 277: 965-968 (August 1997)). Alternatively, standard Southern hybridization techniques can be employed to evaluate gene amplification. For example, Southern analysis is carried out using a non-repetitive fragment of genomic AIB1 DNA, e.g., derived from the 20q11 P1 clone described above or another AIB1 gene-containing genomic clone, as a probe.

The level of gene expression may be measured using methods known in the art, e.g., *in situ* hybridization, Northern blot analysis, or Western blot analysis using AIB1-specific monoclonal or polyclonal antibodies. AIB1 gene transcription was measured using Northern analysis. For example, the data shown in Fig. 2 was obtained as follows. The blot was hybridized sequentially with a probe (ER, AIB1, TIF2, SRC-1, or  $\beta$ -actin as indicated to the left of the photograph). AIB1 expression was compared to that of ER, TIF2, and SRC-1. cDNA clones were obtained from Research Genetics [TIF2 (clone 132364, GenBank accession no. R25318); SRC-1 (clone 418064, GenBank accession no. W90426)], the American Type Culture Collection (pHEGO-hyg, ATCC number 79995), or Clontech ( $\beta$  actin). The AIB1 probe was a 2.2kb NotI/SacI fragment of pCMVSPORT-B11. The  $\beta$ -actin probe was used as a control for loading error. To avoid cross-hybridization between these related genes and to match signal intensities, similar sized probes from

the 3'UTRs of AIB1, TIF2, and SRC-1 were utilized. Each of these probes detected a signal in normal mammary RNA on longer exposure. Electrophoresis, transfer and hybridization of 15  $\mu$ g total RNA was performed by standard methods.

5     **B.     Detection of AIB1 Gene Products**

AIB1 polypeptides to be used as antigens to raise AIB1-specific antibodies can be generated by methods known in the art, e.g., proteolytic cleavage, *de novo* synthesis, or expression of a recombinant polypeptide from the cloned AIB1 gene or a fragment thereof. AIB1-specific antibodies are then produced using standard methodologies for raising polyclonal antisera and making monoclonal antibody-producing hybridoma cell lines (see Coligan et al., eds., *Current Protocols in Immunology*, 1992, Greene Publishing Associates and Wiley-Interscience). To generate monoclonal antibodies, a mouse is immunized with an AIB1 polypeptide, antibody-secreting B cells isolated from the mouse, and the B cells immortalized with a non-secretory myeloma cell fusion partner. Hybridomas are then screened for production of an AIB1-specific antibody and cloned to obtain a homogenous cell population which produces a monoclonal antibody.

For administration to human patients, antibodies, e.g., AIB1 specific monoclonal antibodies, can be humanized by methods known in the art. Antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; Oxford Molecular, Palo Alto, CA).

20     Example 4: Detection of AIB1-related cell proliferative disorders

A.     Diagnostic and Prognostic Methods

The invention includes a method of detecting an aberrantly proliferating cell, e.g., a steroid hormone-responsive cancer cell such as a breast cancer cell, an ovarian cancer cell, colon cancer cell, or prostate cancer cell, by detecting the number of AIB1 gene copies in the cell and/or the level of expression of the AIB1 gene product. AIB1 gene amplification or gene expression in a patient-derived tissue sample is measured as described above and compared to the level of amplification or gene expression in normal non-cancerous cells. An increase in the level of amplification or gene expression detected in the patient-derived biopsy sample compared to the normal control is diagnostic of a diseased state, i.e., the presence of a steroid hormone responsive cancer.

Because of the importance of estrogen exposure to mammary carcinogenesis and of anti-estrogen treatment in breast cancer therapy, such assays are also useful to determine the frequency of alterations of AIB1 expression in pre-malignant breast lesions (e.g. ductal carcinoma *in situ*) and during the progression from hormone dependent to hormone independent tumor growth.

35     The diagnostic methods of the invention are useful to determine the prognosis of a patient and estrogen responsive status of a steroid hormone-responsive cancer.

AIB1 expression can also be measured at the protein level by detecting an AIB1 gene products with an AIB1-specific monoclonal or polyclonal antibody preparation.



## B. Diagnosis of Tamoxifen-Sensitivity

Overexpression of AIB1, e.g., as a result of AIB1 gene amplification, in steroid hormone-responsive cancers can predict whether the cancer is treatable with anti-endocrine compositions, e.g., tamoxifen. AIB1 amplification or overexpression in a patient-derived tissue sample compared to a normal (non-cancerous) tissue indicates tumor progression.

Absence of AIB1, e.g., loss of all or part of the AIB1 gene, but retention of ER-positivity in steroid hormone-responsive cancers predicts failure or poor responsiveness to anti-endocrine therapy, e.g., administration of anti-estrogen compositions such as tamoxifen. Since loss of AIB1 expression in a cancer cell may indicate a disruption of the ER signal transduction pathway, anti-estrogen therapy may be ineffective to treat such cancers. Patients identified in this manner (who would otherwise be treated with anti-estrogens) would be treated with alternative therapies.

Loss of estrogen receptor in recurrent breast cancer is also associated with poor response to endocrine therapy. Up to 30% to 40% of metastases from hormone receptor-positive primary breast cancer do not respond to endocrine therapy. The frequency of hormone receptor status changes between primary and recurrent tumors and whether such a change might explain unresponsiveness to endocrine therapy was examined. Primary breast cancer samples and matched asynchronous recurrences were studied from 50 patients who had not received any adjuvant therapy. ER and progesterone receptor (PR) status was determined immunohistochemically from histologically representative formalin-fixed paraffin-embedded tumor samples. ER status was ascertained by mRNA in situ hybridization. Thirty-five (70%) of 50 primary tumors were positive for ER and 30 (60%) for PR. Hormone receptor status of the recurrent tumor differed from that of the primary tumor in 18 cases (36%). Discordant cases were due to the loss of ER (n=6), loss of PR (n=6), or loss of both receptors (n=6). Receptor-negative primary tumors were always accompanied by receptor-negative recurrences. Among 27 patients with ER-positive primary tumors, loss of ER was a significant predictor ( $P=.0085$ ) of poor response to subsequent endocrine therapy. Only one of eight patients (12.5%) with lost ER expression responded to tamoxifen therapy, whereas the response rate was 74% (14 of 19) for patients whose recurrent tumors retained ER expression. Loss of ER expression in recurrent breast cancer predicts poor response to endocrine therapy in primarily ER-positive patients. Evaluation of ER expression and/or AIB1 expression (or gene copy number) is useful to determine the most effective approach to treatment of steroid-responsive cancers.

### Example 5: Screening of candidate compounds

#### A. *In vitro* assays

The invention includes methods of screening to identify compounds which inhibit the interaction of AIB1 with ER, thereby decreasing estrogen dependent transcription which leads to aberrant cell proliferation. A transcription assay is carried out in the presence and absence of the candidate compound. A decrease in transcription in the presence of the compound compared to that

in its absence indicates that the compound blocks an AIB1/ER interaction and inhibits estrogen dependent transcription.

To determine the effect of AIB1 on estrogen-dependent transcription, an ER reporter plasmid can be used. The transcription assays described herein were conducted as follows. COS-1  
5 cells were grown and maintained in phenol-red free DMEM medium supplemented with 10% charcoal-stripped fetal bovine serum. Cells were plated into 6-well culture dishes at  $1.5 \times 10^5$  cells/well and allowed to grow overnight. Transfection of cells with the ER reporter plasmid was performed with Lipofectamine (Gibco, BRL) following the manufacturer's protocol. Three ng pRL-CMV were used as an internal control for transfection efficiency. Ligand or ethanol vehicle  
10 was added 234 hours post-transfection and cell lysates were harvested 48 hours post-transfection. Reporter activities were determined using the Dual-Luciferase Reporter Assay System (Promega) and the results expressed in relative luminescence units (RLU; luciferase/*Renilla* luciferase). pRL-CMV and pGL3-promoter were obtained from Promega. pHEGO-hyg was obtained from ATCC. The ER reporter pGL3.luc.3ERE contains three tandem copies of the ERE upstream from the SV40  
15 promoter driving the luciferase gene. Standard mammalian expression vectors were utilized. Empty pcDNA3 vector was added to each of the pcDNA3.1-AIB1 dilutions to maintain constant amounts of plasmid DNA.

Compounds which inhibit the interaction of AIB1 with ER are also identified using a standard co-precipitation assay. AIB1/ER co-precipitation assays are carried out as follows. An  
20 AIB1 polypeptide and an ER polypeptide are incubated together to allow complex formation. One of the polypeptides is typically a fusion protein, e.g., GST-AIB1, and the other is tagged with a detectable label, e.g.,  $^{32}\text{P}$ -labeled ER). After incubation, the complex is precipitated, e.g., using glutathione-Sepharose beads. The beads are washed, filtered through a glass fiber filter, and collected. The amount of co-precipitated  $^{32}\text{P}$ -label is measured. A reduction in the amount of co-precipitated label in the presence of a candidate compound compared to that in the absence of the  
25 candidate compound indicates that the compound inhibits an AIB1/ER interaction

Alternatively, a standard *in vitro* binding assay can be used. For example, one polypeptide, e.g., AIB1, can be bound to a solid support and contacted with the second polypeptide, e.g., ER. The amount of the second polypeptide which is retained on the solid support is then measured. A  
30 reduction in the amount of retained (second) polypeptide in the presence of a candidate compound compared to that in its absence indicates that the compound inhibits an AIB1/ER interaction. Techniques for column chromatography and coprecipitation of polypeptides are well known in the art.

An evaluation of AIB1/ER interaction and identification of compounds that blocks or  
35 reduces the interaction can also be carried out *in vivo* using a yeast two-hybrid expression system in which the activity of a transcriptional activator is reconstituted when the two proteins or polypeptides of interest closely interact or bind to one another.

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The yeast GAL4 protein consists of functionally distinguishable domains. One domain is responsible for DNA-binding and the other for transcriptional activation. In the two-hybrid expression system, plasmids encoding two hybrid proteins, a first fusion protein containing the GAL4 DNA-binding domain fused to a first protein, e.g., AIB1, and the second fusion protein containing the GAL4 activation domain fused to a second protein, e.g., ER, are introduced into yeast. If the two proteins are able to interact with one another, the ability to activate transcription from promoters containing Gal4-binding sites upstream from an activating sequence from GAL1 (UAS<sub>G</sub>) is reconstituted leading to the expression of a reporter gene. A reduction in the expression of the reporter gene in the presence of a candidate compound compared to that in the absence of the compound indicates that the compound reduces an AIB1/ER interaction.

A method of identifying a DNA-binding protein which regulates AIB1 transcription can be carried out as follows:

A DNA containing a cis-acting regulatory element can be immobilized on polymeric beads, such as agarose or acrylamide. A mixture of proteins, such as a cell lysate, is allowed to come in contact with and bind to the DNA. Following removal of non-binding proteins, specifically-bound proteins, are eluted with a competing DNA sequence which may be identical to the immobilized sequence. Specific binding of a protein to the DNA regulatory element indicates that the protein may regulate AIB1 transcription. Functional activity of the identified trans-acting factor can be confirmed with an appropriate functional assay, such as one which measures the level of transcription of a reporter gene having the cis-acting regulatory gene 5' to the transcription start site of AIB1.

A method of identifying a compound which decreases the level of AIB1 transcription can be accomplished by contacting an immobilized AIB1-derived cis-acting regulatory element with a trans-acting regulatory factor in the presence and absence of candidate compound. A detectable change, i.e., a reduction, in specific binding of the trans-acting factor to its DNA target indicates that the candidate compound inhibits AIB1 transcription.

In addition to interacting with ER, AIB1 also interacts with the transcriptional integrators CBP and p300. CBP and p300 participate in the basal transcriptional apparatus in a cell. Thus, another approach to inhibit signal transduction through AIB1 is to prevent the formation of or disrupt an interaction of AIB1 with CBP and/or p300. Compounds which inhibit signal transduction (and therefore cell proliferation) can be identified by contacting AIB1 (or a fragment thereof which interacts with CBP or p300) with CBP or p300 (or a fragment thereof containing an AIB1-interacting domain, e.g., a C-terminal fragment) in the presence and absence of a candidate compound. For example, a C-terminal fragment of CBP involved in steroid receptor co-activator interaction contains 105 amino acids in the Q-rich region of CBP (Kamei et al., 1996, Cell 85:403-414; Yao et al., 1996, Proc. Natl. Acad. Sci. USA 93:10626-10631; Hanstein et al., 1996, Proc. Natl. Acad. Sci. USA 93:11540-11545). A decrease in AIB1 interaction with CBP or p300 in the presence of a candidate compound compared to that its absence indicates that the compound inhibits AIB1 interaction with these transcriptional integrators, and as a result, AIB1-mediated signal

transduction leading to DNA transcription and cell proliferation. Compounds which inhibit AIB1 interaction with transcriptional integrators can also be identified using a co-precipitation assay and the yeast two-hybrid expression system described above.

5     **B.     *In vivo* assays**

Transgenic mice are made by standard methods, e.g., as described in Leder et al., U.S. Patent No. 4,736,866, herein incorporated by reference, or Hogan et al., 1986 *Manipulating the Mouse Embryo*. Cold Spring Harbor Laboratory" New York.

10     Briefly, a vector containing a promoter operably linked to AIB1-encoding cDNA is injected into murine zygotes, e.g., C57BL/6J X DBA/2F2 zygotes. Incorporation of the transgene into murine genomic DNA is monitored using methods well known in the art of molecular biology, e.g., dot blotting tail DNA with a probe complimentary to the 3' region of the gene contained in the AIB1 transgene construct. Mice thus confirmed to harbor the transgene can then be used as founders. Animal lines are created by crossing founders with C57BL/6J mice (The Jackson  
15     Laboratory, Bar Harbor, ME). AIB1 transgenic mice can be used to screen candidate compounds *in vivo* to identify compounds which inhibit aberrant cell proliferation, e.g., as measured by reduction tumor growth or metastasis. AIB1 transgenic mice are also useful to identify other genes involved in steroid hormone receptor-dependent cancers and to establish mouse cell lines which overexpress AIB1. AIB1-overexpressing cell lines are useful to screen for compounds that  
20     interfere with AIB1 function, e.g., by blocking the interaction of AIB1 with a ligand.

Example 6: AIB1 therapy

As discussed above, AIB1 is a novel member of the SRC-1 family of transcriptional co-activators. Amplification and overexpression of AIB1 in ER-positive breast and ovarian cancer  
25     cells and in breast cancer biopsies implicate this protein as a critical component of the estrogen response pathway. AIB1 overexpression results in increased ER-dependent transcriptional activity which confers a growth advantage of AIB1 amplification-bearing clones during the development and progression of estrogen-dependent cancers.

Compounds which inhibit or disrupt the interaction of an AIB1 gene product with a steroid  
30     hormone receptor, e.g., ER, are useful as anti-neoplastic agents for the treatment of patients suffering from steroid hormone-responsive cancers such as breast cancer, ovarian cancer, prostate cancer, and colon cancer. Likewise, compounds which disrupt interaction between AIB1 and p300 and/or CBP are also useful as anti-neoplastic agents.

AIB1 polypeptides or peptide mimetics of such polypeptides, e.g., those containing domains  
35     which interact with steroid hormone receptors, can be administered to patients to block the interaction of endogenous intracellular AIB1 and a steroid hormone receptor, e.g., ER in an aberrantly proliferating cell. A mimetic may be made by introducing conservative amino acid substitutions into the peptide. Certain amino acid substitutions are conservative since the old and

the new amino acid share a similar hydrophobicity or hydrophylicity or are similarly acidic, basic or neutrally charged (Stryer "Biochemistry" 1975, Ch.2, Freeman and Company, New York).

Conservative substitutions replace one amino acid with another amino acid that is similar in size, hydrophobicity, etc. Examples of conservative substitutions are shown in the table below (Table 1).

TABLE 1

Original Residue	Conservative Substitutions
Ala	ser
Arg	lys
Asn	gln, his
Asp	glu
Cys	ser
Gln	asn
Glu	asp
Gly	pro
His	asn; gln
Ile	leu, val
Leu	ile; val
Lys	arg; gln; glu
Met	leu; ile
Phe	met; leu; tyr
Ser	thr
Thr	ser
Trp	tyr
Tyr	trp; phe
Val	ile; leu

Variations in the cDNA sequence that result in amino acid changes, whether conservative or not, should be minimized in order to preserve the functional and immunologic identity of the encoded protein.

Compositions administered therapeutically include polypeptide mimetics in which one or more peptide bonds have been replaced with an alternative type of covalent bond which is not susceptible to cleavage by peptidases. Where proteolytic degradation of the peptides following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a noncleavable peptide mimetic yields a more stable and thus more useful therapeutic polypeptide.

Such mimetics, and methods of incorporating them into polypeptides, are well known in the art. Similarly, the replacement of an L-amino acid residue with a D-amino acid residue is a standard way of rendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl, succinyl, methoxysuccinyl, suberyl, adipyl, azelalyl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelalyl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl.

AIB1 polypeptides or related peptide mimetics may be administered to a patient intravenously in a pharmaceutically acceptable carrier such as physiological saline. Standard methods for intracellular delivery of peptides can be used, e.g. packaged in liposomes. Such methods are well known to those of ordinary skill in the art. It is expected that an intravenous dosage of approximately 1 to 100  $\mu$ moles of the polypeptide of the invention would be administered per kg of body weight per day. The compositions of the invention are useful for parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal.

The therapeutic compositions of this invention may also be administered by the use of surgical implants which release the compounds of the invention. These devices could be readily implanted into the target tissue, e.g., a solid tumor mass, and could be mechanical or passive. Mechanical devices, such as pumps, are well known in the art, as are passive devices (e.g., consisting of a polymer matrix which contains therapeutic formulations; these polymers may slowly dissolve or degrade to release the compound, or may be porous and allow release via pores).

Antisense therapy in which a DNA sequence complementary to an AIB1 mRNA transcript is either produced in the cell or administered to the cell can be used to decrease AIB1 gene expression thereby inhibiting undesired cell proliferation, e.g., proliferation of steroid hormone-responsive cancer cells. An antisense polynucleotide, i.e., one which is complementary of the coding sequence of the AIB1 gene, is introduced into the cells in which the gene is overproduced. The antisense strand (either RNA or DNA) may be directly introduced into the cells in a form that is capable of binding to the transcripts. Alternatively, a vector containing a DNA sequence which, once within the target cells, is transcribed into the appropriate antisense mRNA, may be administered. An antisense nucleic acid which hybridizes to the coding strand of AIB1 DNA can decrease or inhibit production of an AIB1 gene product by associating with the normally single-stranded mRNA transcript, and thereby interfering with translation.

DNA is introduced into target cells of the patient with or without a vector or using standard vectors and/or gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, and adenoviruses, among others. The DNA of the invention may be administered in a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal e.g., physiological saline. A therapeutically effective amount is an amount of the nucleic acid of the invention which is capable of producing a medically desirable result in a patient. As is well known in the medical arts, dosage for any given patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary, but a preferred dosage for intravenous administration of a nucleic acid is from approximately  $10^6$  to  $10^{22}$  copies of the nucleic acid molecule.

Determination of optimal dosage is well within the abilities of a pharmacologist of ordinary skill.

Example 7: AIB1 Knockout and Overexpression Mouse Mutants

5 Mutants organism that underexpress or overexpress AIB1 are useful for research. Such mutants allow insight into the physiological and/or pathological role of AIB1 in a healthy and/or pathological organism. These mutants are said to be "genetically engineered," meaning that information in the form of nucleotides has been transferred into the mutant's genome at a location, or in a combination, in which it would not normally exist. Nucleotides transferred in this way are  
10 said to be "non-native." For example, a WAP promoter inserted upstream of a native AIB1 gene would be non-native. An extra copy of a mouse AIB1 gene present on a plasmid and transformed into a mouse cell would be non-native. Mutants may be, for example, produced from mammals, such as mice, that either overexpress AIB1 or underexpress AIB1 or that do not express AIB1 at all. Overexpression mutants are made by increasing the number of AIB1 genes in the organism, or  
15 by introducing an AIB1 gene into the organism under the control of a constitutive or inducible or viral promoter such as the mouse mammary tumor virus (MMTV) promoter or the whey acidic protein (WAP) promoter or the metallothionein promoter. Mutants that underexpress AIB1 may be made by using an inducible or repressible promoter, or by deleting the AIB1 gene, or by destroying or limiting the function of the AIB1 gene, for instance by disrupting the gene by transposon  
20 insertion.

Anti-sense genes may be engineered into the organism, under a constitutive or inducible promoter, to decrease or prevent AIB1 expression. A gene is said to be "functionally deleted" when genetic engineering has been used to negate or reduce gene expression to negligible levels. When a mutant is referred to in this application as having the AIB1 gene altered or functionally  
25 deleted, this reference refers to the AIB1 gene and to any ortholog of this gene, for instance "a transgenic animal wherein at least one AIB1 gene has been functionally deleted" would encompass the mouse ortholog of the AIB1 gene, pCIP. When a mutant is referred to as having "more than the normal copy number" of a gene, this means that it has more than the usual number of genes found in the wild-type organism, eg: in the diploid mouse or human.

30 A mutant mouse overexpressing AIB1 may be made by constructing a plasmid having the AIB1 gene driven by a promoter, such as the mouse mammary tumor virus (MMTV) promoter or the whey acidic protein (WAP) promoter. This plasmid may be introduced into mouse oocytes by microinjection. The oocytes are implanted into pseudopregnant females, and the litters are assayed for insertion of the transgene. Multiple strains containing the transgene are then available for  
35 study.

WAP is quite specific for mammary gland expression during lactation, and MMTV is expressed in a variety of tissues including mammary gland, salivary gland and lymphoid tissues.

Many other promoters might be used to achieve various patterns of expression, e.g., the metallothionein promoter.

An inducible system may be created in which AIB1 is driven by a promoter regulated by an agent which can be fed to the mouse such as tetracycline. Such techniques are well known in the art.

A mutant knockout mouse from which the AIB1 (also called pCIP) gene is deleted was made by removing coding regions of the AIB1 gene from mouse embryonic stem cells. Fig. 5 shows the intron/exon structure for pCIP. Using this table, mutations can be targeted to coding sequences, avoiding silent mutations caused by deletion of non-coding sequences. (Fig. 6 shows the intron/exon structure for the human AIB1 gene). These cells were microinjected into mouse embryos leading to the deletion of the mouse AIB1 gene in the germ line of a transgenic mouse. The mouse AIB1 gene was mapped and isolated by the following method: The primers AIB/mEST F1

(5'-TCCTTTTCCCAGCAGCAGTTTG-3'; SEQ.I.D. 10) and AIB1/mEST R1 (5'-ATGCCAGACATGGGCATGGG-3' SEQ.I.D.11) were used to screen a mouse Bacterial Artificial Chromosome (BAC) library and to isolate a mouse BAC (designated 195H10). This BAC was assigned to mouse chromosome 2 by fluorescence in situ hybridization (FISH). This region is the mouse equivalent of the portion of human chromosome 20 which carries AIB1.

To map the structure of the gene, first the structure of the human AIB1 gene was determined by polymerase chain reaction of a human genomic DNA clone containing AIB1 using standard methods (Genomics 1995 Jan 20;25(2):501-506) and then the sequences of the intron exon boundaries were determined (Fig.4). Based on this information, the corresponding regions of the mouse BAC were sequenced. The structure of the mouse gene corresponds closely to that of the human gene (Fig. 4). This information localizes the coding regions of the mouse AIB1 gene so that a targeting vector can be constructed to remove these regions from mouse embryonic stem cells. These cells can be then injected into mouse embryos leading to deletion of the mouse AIB1 gene in the germ line of a transgenic mouse. The methods of creating deletion mutations by using a targeting vector have been described in Cell ( Thomas and Capecch, Cell 51(3):503-512, 1987).

References and patents referred to herein are incorporated by reference.

The above examples are provided by way of illustration only and are in no way intended to limit the scope of the invention. One of skill in the art will see that the invention may be modified in various ways without departing from the spirit or principle of the invention. We claim all such modifications.